Elevated expression of membrane type 1 metalloproteinase (MT1-MMP) in reactive astrocytes following neurodegeneration in mouse central nervous system

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Abstract Reactive astrocytes occurring in response to neurodegeneration are thought to play an important role in neuronal regeneration by upregulating the expression of extracellular matrix (ECM) components as well as the ECM degrading metalloproteinases (MMPs). We examined the mRNA levels and cellular distribution of membrane type matrix metalloproteinase 1 (MT1-MMP) and tissue inhibitors 1–4 of MMPs (TIMPs) in brain stem and spinal cord of wobbler (WR) mutant mice affected by progressive neurodegeneration and astrogliosis. MT1-MMP, TIMP-1 and TIMP-3 mRNA levels were elevated, whereas TIMP-2 and TIMP-4 expression was not affected. MT1-MMP was expressed in reactive astrocytes of WR. In primary astrocyte cultures, MT1-MMP mRNA was upregulated by exogeneous tumor necrosis factor α. Increased plasma membrane and secreted MMP activities were found in primary WR astrocytes.

Key words: Neurodegeneration; Wobbler mouse; Astrocyte; Membrane type matrix metalloproteinase 1; Tissue inhibitor of metalloproteinase; Tumor necrosis factor α

1. Introduction

Astrocyte activation, termed reactive gliosis, is a stereotype reaction of astrocytes to neuronal injury leading to tissue remodelling and the formation of a glial scar (reviewed in [1]). A prerequisite for extracellular matrix (ECM) remodelling after neuronal injury is the expression of members of the family of zinc-dependent matrix metalloproteinases (MMP) by neurons and astrocytes (reviewed in [2]). Proteinase activity in turn is regulated by the balance between MMPs, membrane type MMPs (MT-MMPs) and by tissue inhibitors of metalloproteinases (TIMPs) (reviewed in [3]). In models proposed by Deryugina et al. [4] and Lehti et al. [5], a ternary complex consisting of MT1-MMP, TIMP-2 and pro-MMP-2 is generated on the cell surface. Overexpression of MT1-MMP results in increased pro-MMP-2 processing and subsequent shedding, whereas active MMP-2 becomes rebound to the plasma membrane by binding to $\alpha_v\beta_3$ integrin [6]. In non-neuronal cells, the expression of MT1-MMP can be upregulated by tumor necrosis factor α (TNF-α), indicating its involvement in inflammatory processes [7].

One of the hallmarks of the hereditary wobbler (WR) disease (*wr*, chromosome (Chr.) 11) [8] of the mouse is hypertrophy of reactive astrocytes and microglia activation in the white and gray matter of the spinal cord (SC) as indicated by increased expression of glial fibrillary acidic protein (GFAP) and CD45, respectively [9,10]. WR mice are characterized by progressive neurodegeneration in the thalamus (*N. ventralis*), in deep cerebellar nuclei, brain stem (BS) (*N. vestibularis*), SC interneurons and large motoneurons [10]. In the course of the disease, the progressive degeneration of motoneurons results in muscular atrophy resembling human neurodegenerative diseases like spinal muscular atrophy and amyotrophic lateral sclerosis [11]. Prior to the onset of astrogliosis, degenerating motoneurons express pro-transforming growth factor α (TGFα) followed by the TGFα/epidermal growth factor (EGF) receptor expression in reactive astrocytes [12]. Since EGF, which is homologous to TGFα, induces proliferation and differentiation in cultured astrocytes, it has been speculated that TGFα stimulates astrocyte differentiation in the WR disease. We and others have described the induction of proinflammatory cytokines interleukin-1β and TNF-α in SC and BS of WR mice [13,14]. However, the target genes of these cytokines in WR mice were unknown. Therefore, we investigated the expression of MT1-MMP in mouse mutants affected by neurodegeneration. In particular, we analyzed the mechanisms regulating proteinase activity under pathological conditions in WR mice.

2. Materials and methods

2.1. Materials

Recombinant mouse TNF-α was obtained from Boehringer Bioproducts (Mannheim, Germany), dissolved in sterile phosphate-buffered saline (PBS; 140 mM NaCl, 10 mM Na, K phosphate, pH 7.3) to a concentration of 5 ng/μl (300 U) and kept in aliquots at −20°C.

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Abbreviations: CNS, central nervous system; ECM, extracellular matrix; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein; MDF, muscle deficient; MMP, matrix metalloproteinase; MND2, motoneuron disease 2; MT-MMP, membrane type MMP; TGFα, transforming growth factor α; TIMP, tissue inhibitor of metalloproteinase; TNF-α, tumor necrosis factor α; WR, wobbler; WT, wild-type

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2.2. Mouse strains

The mouse mutations '*wobbler*' (*wr*, WR, Chr. 11) [8,11], '*motoneuron disease*' 2 (*mnd2*, MND2, Chr. 6; [15]) and '*muscle deficient*' (*mdf*, MDF, Chr. 19) [16–18] were kept on a C57BL/6 background. For all analyses, we used 25–60 day old genotyped WR (*wr*/*wr*) or wild-type (WT) (+/+) [10] individuals or in the case of MND2 and MDF mutant mice, animals with a manifest phenotype and their normal control littermates. Breeding and experimental use of mice were performed in agreement with the German law on the protection of animals, with a permit by the local authorities to H.J.

2.3. Cell lines

HEK293 (ACC 305) and HT1080 (ACC 315) cells were obtained from the German Collection of Microorganisms and Cell Culture DSMZ (Braunschweig, Germany) and propagated in DMEM/10% fetal calf serum (FCS). MT1-MMP-transfected HEK293 cells were kindly provided by Dr. C. Mauch (Cologne, Germany).

2.4. Tissue preparation

For RNA preparation and histological analyses, mice were killed by decapitation. The central nervous system (CNS) was dissected into cerebrum (Cbr), comprising telencephalon and diencephalon, and into cerebellum (Cbl), BS and cervical SC with cervical segments C1–C8. For cryosections, parts of the CNS were mounted in freezing medium and rapidly shock frozen in propane/liquid nitrogen.

2.5. Generation of MT1-MMP specific antibodies

Polyclonal antibodies reactive for MT1-MMP were raised in rabbits against the synthetic peptides PPGDLRTHTQRSPQSLS(C) and CDGNFDTVAMLRGEM corresponding to the propeptide (α-PD) and hemopexin-like domains (α-HD), respectively (Fig. 2A). The peptides were conjugated to keyhole limpet hemocyanin via an additional cysteine residue.

2.6. Preparation of plasma membrane-enriched cell fractions

CNS tissue samples (300 mg) were homogenized in 4 ml 8.7% (w/v) sucrose in 20 mM Tris–HCl pH 7.4 containing proteinase inhibitors ('complete EDTA-free'; Roche, Mannheim, Germany) on ice and loaded onto 9 ml of 38% (w/v) sucrose in 20 mM Tris–HCl pH 7.4. Following centrifugation at $150\,000\times g$ for 1 h at 4°C, the enriched plasma membrane fraction was isolated from the interphase and diluted in 8.7% (w/v) sucrose solution. The plasma membranes were pelleted again at $150\,000\times g$ for 1 h at 4°C, resuspended in 68 mM Tris–HCl pH 6.8 and stored at −70°C. The amount of protein in the plasma membrane preparation was estimated according to Bradford (modified, [19]).

2.7. Immunoblotting

30 μg of enriched plasma membrane fractions were solubilized in Laemmli buffer at room temperature, subsequently boiled and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [20]. Proteins were transferred to nitrocellulose by electroblotting. Following blocking with PBS/5% non-fat dry milk, the membrane was incubated with 10 μg/ml of rabbit anti-MT1-MMP-HD or anti-MT1-MMP-PD for 16 h at 4°C. After extensive washing, the membrane was incubated with goat anti-rabbit IgG coupled to horseradish peroxidase and developed with the ECL Western blot detection kit (Amersham Pharmacia Biotech, Heidelberg, Germany).

2.8. Immunocytochemistry

Cryosections of CNS tissues, usually 10 μm in thickness, were fixed for 5 min with ice-cold methanol or acetone, washed three times with PBS and blocked with PBS/0.5% bovine serum albumin (BSA). For double labeling, cryosections were incubated with MT1-MMP specific rabbit antibodies (10 μg/ml in PBS/0.5% BSA) and with a monoclonal Cy3-conjugated anti-GFAP antibody (1:800; Sigma, Deisenhofen, Germany) or Cy3-conjugated anti-CD45 (1:200, clone 30F11.1, Pharmingen), followed by the incubation with goat-anti rabbit Cy2-conjugated antibodies (1:500; Dianova, Hamburg, Germany).

2.9. Astrocyte cultures

CNS from animals killed by decapitation was removed immediately and dissected into Cbl, BS and SC. Samples were kept in HEPES-buffered saline (pH 7.6) and mechanically dissociated with scissors. The homogenates were further minced by triturating with an 18 gauge needle and incubated for 10 min with 0.25% trypsin/EDTA (Gibco BRL, Karlsruhe, Germany). After centrifugation, the cell pellet was resuspended in astrocyte basal medium (Clonetics, La Jolla, CA, USA) containing 5% FCS and insulin (1 μg/ml). Cells were seeded onto type I collagen (Sigma) coated coverslips. Medium was changed every second day and cell cultures were used for all assays usually after 7 days. The proportions of astrocytes in cell preparations were determined by anti-GFAP staining. Preparations with ≥90% $GFAP^+$ cells were used for all further experiments.

2.10. Reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA from tissues was purified according to Chomczynski and Sacchi [21]. For mRNA isolation, total RNA was further purified by the Dynabead method (Dynal, Hamburg). From primary cell cultures, total RNA was isolated by the RNeasy preparation method (Qiagen, Hilden, Germany). For RT-PCR, either 1 μg of total RNA or 100 ng of mRNA in 20 μl was reverse-transcribed using SuperScript reverse transcriptase (Life Technologies GmbH, Eggenstein, Germany). 0.2 μl of the RT mixture was used for PCR amplification in a total volume of 20 μl containing 1 μmol of each primer (see Table 1) and 'Master-Mix' (Qiagen, Hilden, Germany). Primers were obtained from TIB MolBiol (Berlin, Germany) and are listed in Table 1. Semi-quantitative PCRs were performed by applying a varying number of the following PCR cycles (10–20 cycles): 4 min 94°C (pre-PCR), followed by 1 min at the annealing temperature, 1 min 72°C and 1 min 92°C. The resulting amplification products (see Table 1) were separated by electrophoresis on 2% agarose gels and visualized with ethidium bromide. Ethidium bromide-stained amplification products were blotted onto nitrocellulose, hybridized with a [α-^{32}P]dATP random prime labeled gene specific probe and quantified with a Bio-Imager (Bio-Rad, Göttingen, Germany). Probes were obtained by cloning the respective PCR fragments in pTOPO (Invitrogen) and verified by DNA sequencing. Quantification and analysis of the RT-PCR products were performed on the basis of the standard log plots of hybridization signals versus cycle number (data not shown). RT-PCR experiments were usually performed with 20 cycles, the cDNAs were standardized to an equal level of cDNA for the housekeeping gene L7 [22].

2.11. Immunolocalization of MT1-MMP on cultured astrocytes

After 7 days in culture, primary astrocytes were used for immuno-

Table 1
Primers used for RT-PCR analyses of MT1-MMP, TIMPs 1–4 and L7

Name	Orientation	Sequence	Length (bp)	Temperature (°C)
MT1-MMPmet	sense	5′-CCTCGCTGTGGTGTTCCGGAT-3′	458	64
	antisense	5′-ATGCCCCAACTCATGCAGAGC-3′		
TIMP-1	sense	5′-CCTTTGCATCTCTGGCATCTGGCA-3′	437	68
	antisense	5′-CCAACAGCCAGCACTATAGGTGTT-3′		
TIMP-2	sense	5′-GGACCTGACAAAGACATCGAGTT-3′	348	68
	antisense	5′-TCTCTTGATGCAGGCGAAGAAC-3′		
TIMP-3	sense	5′-CCCTTTGGCACTCTGGTCTACA-3′	374	68
	antisense	5′-TTTGGACTGATAGCCAGGGTACC-3′		
TIMP-4	sense	5′-CCAGTGAGAAAGTAGTCCCTG-3′	461	62
	antisense	5′-GCTGCAGATGCCATCAACATG-3′		
L7	sense	5′-AGATGTACCGCACTGAGATCC-3′	352	62
	antisense	5′-ACTTACCAAGAGACCGAGCAA-3′		

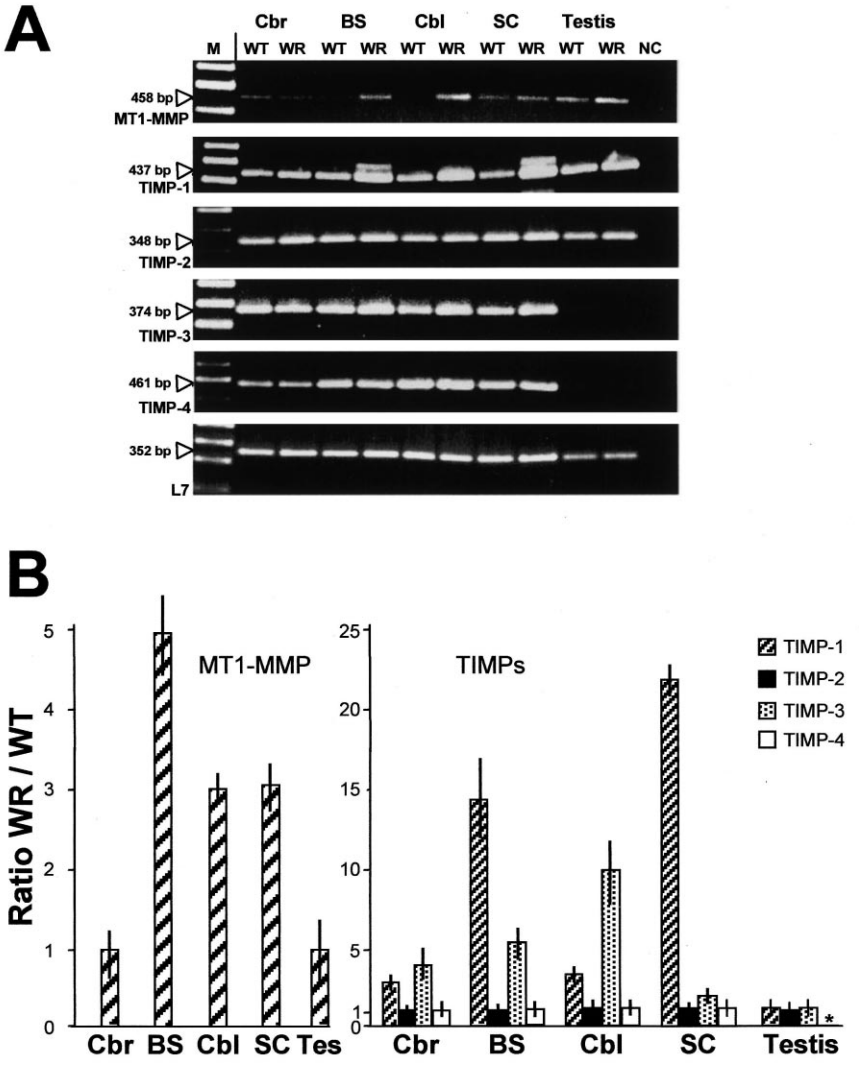

Fig. 1. mRNA levels of MT1-MMP and TIMPs 1–4 in different CNS regions and in testis of WT and WR mice. A: Ethidium bromide-stained gel of PCR products following RT and amplification for 30 cycles. The mRNA for L7 ribosomal protein was used as reference (for sizes of amplificates, see Table 1). NC, control (no template). B: mRNA levels (20 cycles) in WR as compared to WT mice. The amount of radioactivity in imager units normalized to the L7 reference signal is given as ratio WR to WT. Signals are the mean values of four independent experiments. Asterisk, no signal detected. Abbreviations used: Cbr, cerebrum; BS, brain stem; Cbl, cerebellum; SC, spinal cord; Tes, testis.

fluorescence microscopy studies. Cells were rinsed extensively with PBS/0.5 mM $CaCl_2$ and fixed with PBS/2% (w/v) paraformaldehyde at room temperature. After incubation with PBS/0.5 mM $CaCl_2$/2% (w/v) BSA for 45 min at 4°C, cells were preincubated with the rat monoclonal anti-FcγRII specific antibody 2.4G2 (25 μg/ml) for 30 min at 4°C [23]. Samples were incubated with mouse monoclonal anti-MT1-MMP antibody 113-5B7 (40 μg/ml; Fuji Chemical Industries) [24] in the presence of 2.4G2 for 2 h at 4°C. After washing with PBS/0.5 mM $CaCl_2$, cells were incubated with Cy2-conjugated goat anti-mouse polyclonal antibody (Dianova, Hamburg, Germany) for 1 h, washed again and mounted on glass slides with Mowiol 4-88 (Hoechst).

2.12. Determination of MMP activity

To determine the activity of astrocyte plasma membrane-associated and -secreted MMPs, 7 day old astrocyte cultures grown on 3.5 cm tissue culture dishes were washed and incubated for 12 h in 2.5 ml HEPES-buffered saline containing 5 mM glucose. The conditioned medium obtained from 5×10^5 cells was collected and immediately analyzed. Cells were detached with PBS containing 10 mM glucose and resuspended in 2.5 ml 10 mM HEPES buffer. Supernatant and cells were incubated with 500 nmol of the fluorogenic peptide Mca-Pro-Leu-Gly-Leu-Dap-(Dnp)-Ala-Arg-NH_2 (Bachem, Heidelberg) for 1 h at 37°C in the dark, in a total volume of 2.5 ml [25]. Subsequently, cells were pelleted and the supernatant was analyzed on a Perkin Elmer Luminescence Spectrometer L55OB. The proteolytic activity towards the Gly-Leu bond was determined as an increase in the fluorescence intensity following excitation at 328 nm and detection of emission at 393 nm.

3. Results

3.1. Levels of mRNAs for MT1-MMP and TIMP-1, 2, 3 and 4 in the CNS of WT and WR mice

Total RNA from Cbr, BS, Cbl, SC and testis (as non-CNS control tissue) of 40 day old WT and WR mice was subjected to RT-PCR with specific primer pairs (Table 1 and Fig. 1A). Using 30 PCR cycles, MT1-MMP expression was detectable in all CNS regions of WR mice, whereas expression was low in Cbr, BS and SC of WT controls (Fig. 1A). Amplification under non-saturating conditions (20 cycles) revealed altered mRNA levels of MT1-MMP and TIMP-1–4 in affected WR mice and are given as the ratio of the amounts of amplificates,

WR to WT (Fig. 1B). MT1-MMP expression in WR mice was upregulated predominantly in BS, Cbl and SC, whereas expression in the Cbr and testis remained unchanged. Maximal upregulation of the MT1-MMP transcript was observed in the BS (about five-fold). The highest expression of MT1-MMP was found in most severely affected CNS regions in WR mice.

In WR mice, elevated mRNA levels were also found with TIMP-1 and TIMP-3, whereas the mRNA levels of TIMP-2 and TIMP-4 were unaffected by the disease. TIMP-1, TIMP-2 and TIMP-3 expression was detectable in all tissues tested (Fig. 1A). TIMP-4 was highly expressed in CNS, but undetectable in testis (Fig. 1A). TIMP-1 expression was 15-fold increased in BS and 23-fold in SC. Likewise, we observed a tissue-dependent increase in TIMP-3 expression, with highest expression in WR mouse Cbl (12-fold increase, Fig. 1C).

3.2. Increased MT1-MMP protein level in the CNS of the WR mouse

Two polyclonal antibodies reactive to the propeptide (α-PD) and hemopexin-like domain (α-HD) of MT1-MMP were raised in rabbits (Fig. 2A). Plasma membrane-enriched protein extracts from WT and WR CNS were prepared and probed with α-HD (Fig. 2B). As a control, plasma membrane extracts from untransfected and MT1-MMP-transfected HEK293 cells and HT1080 cells were probed with α-HD. The antibody predominantly recognized a 60 kDa band in plasma membrane preparations from MT1-MMP expressing HEK293 cells, HT1080 cells and CNS extracts. The immunoblot also revealed staining of lower M_r proteins that may be degradation products of the 60 kDa protein. The antibody also reacted with a 63 kDa protein in plasma membrane extracts from HT1080 cells which corresponds to the unprocessed form as indicated by probing with α-PD. The α-PD further reacted with a protein of approximately 67 kDa, that may be a precursor form of mature MT1-MMP. Like mRNA levels, protein levels of MT1-MMP were higher in WR than in WT CNS. The predominant form expressed in both WT and WR CNS was the 60 kDa form, i.e. the catalytically active MT1-MMP, whereas faint staining was observed for proteins of 63 kDa and 67 kDa.

3.3. MT1-MMP is expressed in reactive astrocytes

To determine the cellular expression pattern of MT1-MMP SC cryosections of WT and WR mice were probed with MT1-MMP specific antibodies (Fig. 3A–E) and anti-GFAP monoclonal antibody (Fig. 3E,F). Strong GFAP immunostaining was seen with SC sections of 60 day old WR mice but not in age-matched WT mice (Fig. 3E,F, respectively), indicating a strong astrogliosis [9]. Both MT1-MMP specific antibodies, α-PD and α-HD, intensely stained the gray matter in SC sections of WR mice (Fig. 3B,D), whereas WT mice revealed only occasional staining (Fig. 3A,C). MT1-MMP α-PD and α-HD positive cells displayed the typical morphological aspects of astrocytes with long branched processes. Comparison of the number of cells stained with α-PD or α-HD revealed a lower amount of α-PD immunoreactive cells indicating the expression of active MT1-MMP. MT1-MMP immunoreactive cells from WR SC were identified as astrocytes by double immunohistochemical staining with α-GFAP and α-HD (Fig. 3, lower panel). Microglia identified by α-CD45 staining were morphologically distinct from astrocytes and revealed only occasional faint staining with α-HD in SC sections from WR mice (Fig. 3, lower panel).

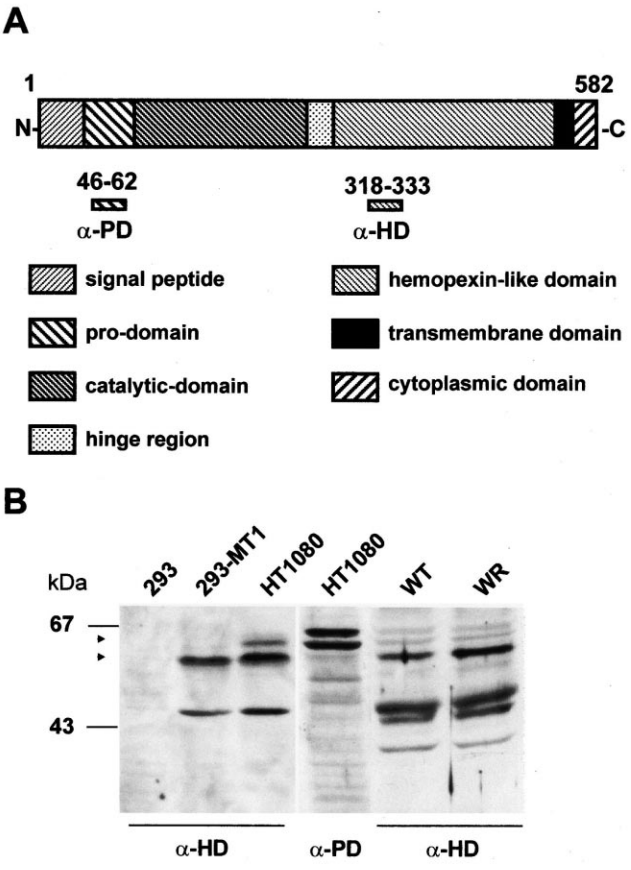

Fig. 2. Generation of MT1-MMP specific antibodies and immunodetection of MT1-MMP in Western blots. A: Domain organization of MT1-MMP and schematic representation of the immunogenic peptides used to raise the polyclonal pro-domain (α-PD) and the hemopexin-like domain (α-HD) antibodies. Amino acid numbering refers to the deduced amino acid sequence of human MT1-MMP beginning with the proposed start methionine (GenBank accession No. D26512). B: Antigen signals of MT1-MMP in CNS plasma membrane preparations from WT and WR mice. Plasma membrane-enriched protein extracts (30 μg per lane) were separated by 10% SDS–PAGE, electroblotted and probed with α-HD. Plasma membrane extracts of HEK293 cells (293), MT1-MMP-transfected HEK293 cells (293-MT1) and HT1080 were used as controls and probed with α-HD. HT1080 protein extracts were further probed with α-PD to determine the identity of the 63 kDa form. Arrows indicate MT1-MMP immunoreactive proteins. The upper arrow corresponds to latent MT1-MMP (63 kDa), the lower arrow to the active form (60 kDa) identified by incubating HT1080 protein extracts with α-PD.

3.4. MT1-MMP expression is elevated in the CNS of mouse mutants with neurodegeneration

To demonstrate the coincidence of MT1-MMP expression with the occurrence of reactive astrocytes in the SC, we stained SC sections of two additional mouse mutants, MDF (*mdf*) [16–18] and MND2 (*mnd2*) [15], with α-HD. In these like in WR, degeneration of motoneurons in the ventral horn is accompanied by local activation of astrocytes as judged by α-GFAP staining. In both mutants, reactive astrocytes express MT1-MMP (Fig. 4). This finding supports the notion that upregulation of MT1-MMP is a common feature of reactive astrocytes responding to neurodegeneration.

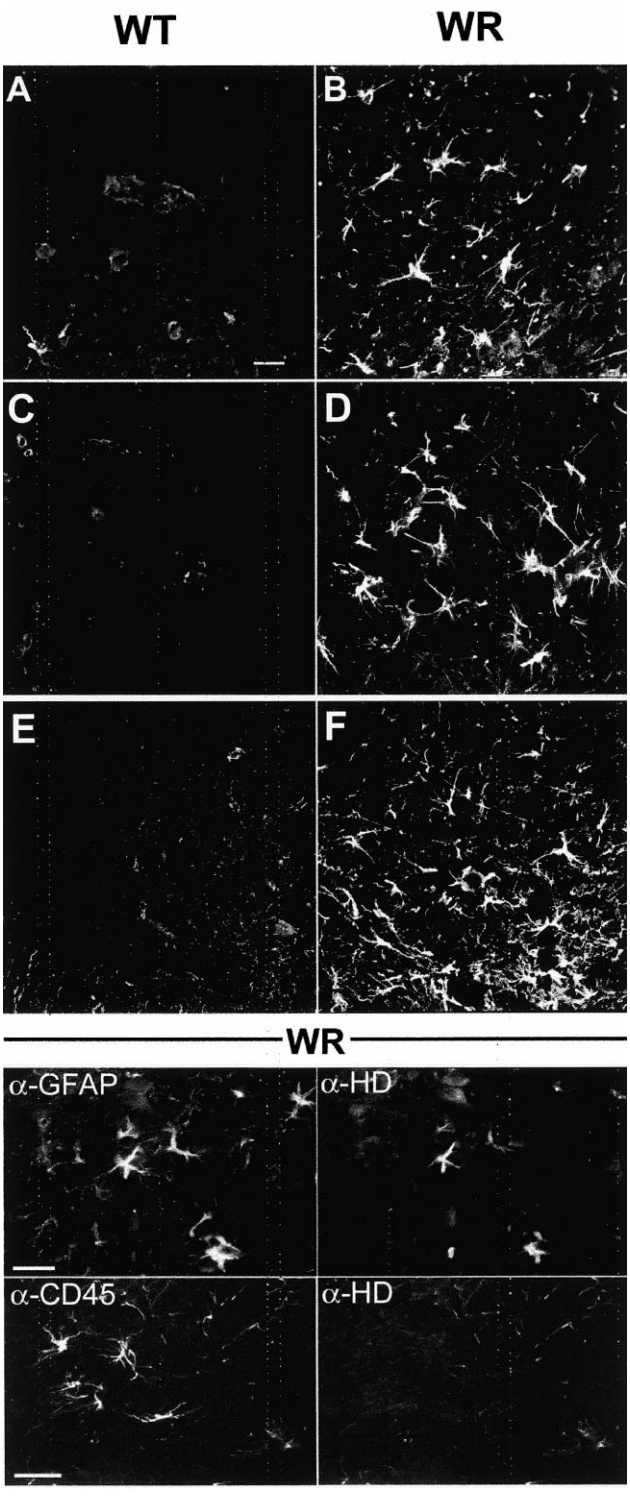

Fig. 3. MT1-MMP in the SC of WT (left, A, C, E) and WR (right, B, D, F) mice. Cervical SC sections (ventral horn) from 60 day old mice were stained with antibodies reactive for the pro-domain (α-PD, A and B) and the hemopexin-like domain (α-HD, C and D). For identification of astrocytes, sections were stained in parallel with anti-GFAP antibody (E and F). For cell type identification, WR SC sections were double-stained for GFAP/MT1-MMP (α-HD) or CD45/MT1-MMP (α-HD). Bar in A, 10 μm, valid for A to F; bars in lower panel, 25 μm.

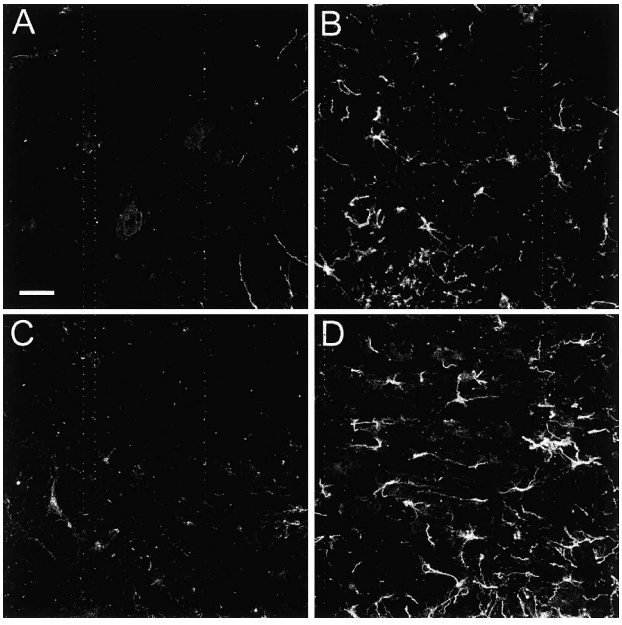

Fig. 4. Immunohistochemical staining of MT1-MMP with α-HD in mouse mutants with motoneuron degeneration in the ventral horn of the SC (white matter to the right). A: WT (C57BL/6), 40 days; B: MDF mutant, 40 days; C: WT (C57BL/6), 30 days; D: MND2 mutant, 30 days. Parallel stainings with anti-GFAP antibody (not shown) revealed that the labeled cells are reactive astrocytes. Bar in A, 30 μm.

3.5. *MT1-MMP and MMP upregulation in primary astrocyte cultures from WR mice*

To determine the expression pattern of TIMPs and MT1-MMP in astrocytes, primary astrocytes derived from affected CNS regions of WT and WR mice were cultured and analyzed during a 7 day culture period in which the cells developed extensive branches (Fig. 5C). Astrocytes from WT and WR mice express TIMP-2 as shown by semi-quantitative RT-PCR, whereas the expression of TIMP-1 and TIMP-3 was relatively low (Fig. 5A,B). This result implies that astrocytes may not be the main source of TIMP-1 and TIMP-3 in SC extracts. Since primary astrocytes from rat brain reportedly express TIMP-1 and TIMP-3, regional effects may influence the repertoire of TIMPs expressed [26]. Despite high expression levels of TIMP-4 in SC, primary astrocyte cultures did not express it (Fig. 5A). A 10-fold upregulation was found for MT1-MMP mRNA levels in primary astrocytes derived from WR as compared to control mice (Fig. 5A,B).

To analyze the cellular localization of MT1-MMP in primary astrocytes, cells attached to collagen type I coated coverslips were stained with MT1-MMP specific mouse monoclonal antibody 113-5B7 [24]. In WT astrocytes, MT1-MMP was not detectable (data not shown). MT1-MMP immunofluorescence staining of WR astrocytes resulted in a speckled labeling of the plasma membrane along the astrocyte processes (Fig. 5C). Using the MMP peptide substrate, Mca-Pro-Leu-Gly-Leu-Dap-(Dnp)-Ala-Arg-NH_2 [25], we found a significant increase in cell-bound and secreted proteolytic activity in primary astrocytes from WR mice (Fig. 5D).

3.6. *TNF-α inducible MT1-MMP expression in primary astrocytes*

As demonstrated for HT1080 cells, the proinflammatory

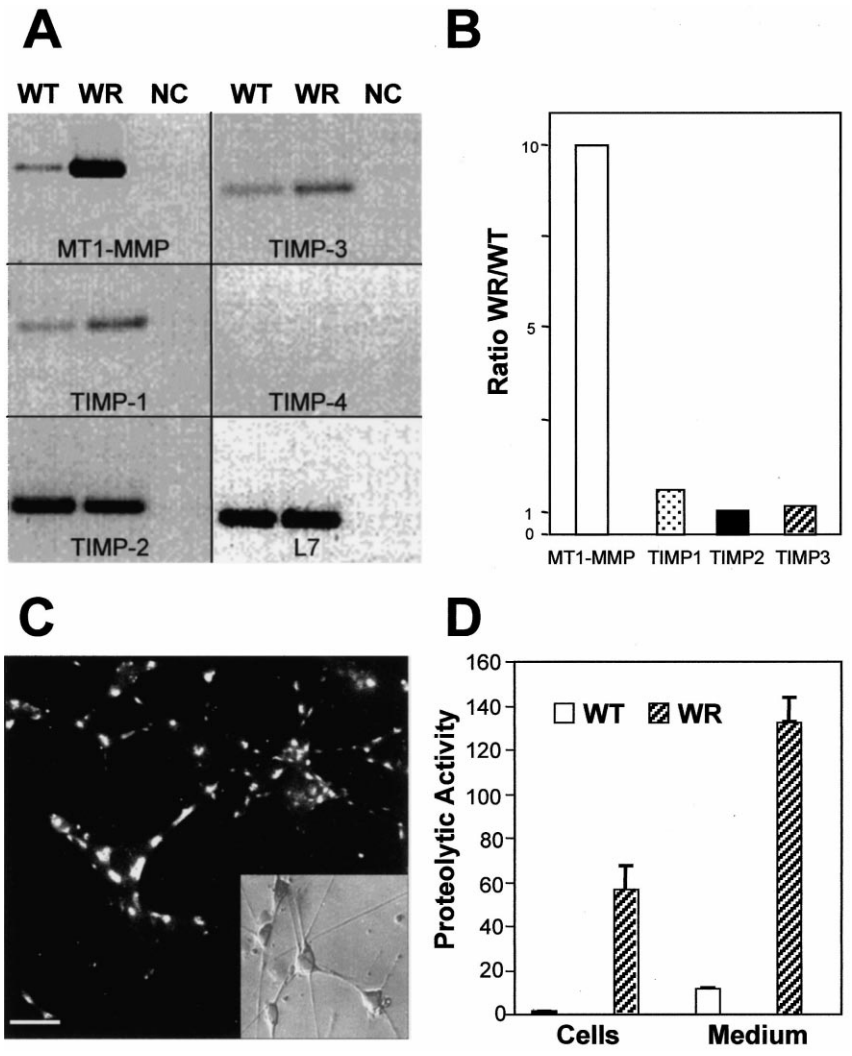

Fig. 5. Expression of MT1-MMP and TIMP-1–4 in primary astrocyte cultures from WT and WR mice. A: mRNA levels of MT1-MMP, TIMP-1–4 and L7 were analyzed as described in Section 2. NC, without template. B: Evaluation of MT1-MMP and TIMP mRNA levels expressed as WR to WT ratio. C: Seven day old astrocyte culture from WR mice fixed with paraformaldehyde and stained with MT1-MMP specific monoclonal antibody 113-5B7 (insert in C, phase contrast w/o fixation). Bar in C, 50 μm. D: Cell-associated ('cells') and secreted ('medium') proteolytic activity in primary astrocyte cultures determined by cleavage of the fluorogenic Mca-peptide. Fluorescence intensity units (excitation at 328 nm, emission at 393 nm) after background subtraction (Mca-peptide incubated without protein). Data from two independent experiments in each of which cultures were pooled preparations from three WR and from three WT mice. Error bars represent the maximum deviation from the mean.

cytokine TNF-α is an important regulator of MT1-MMP expression [7]. As TNF-α is one of the major cytokines induced in affected CNS regions of WR mice [13], we determined mRNA levels of MT1-MMP in WT and WR astrocyte cultures by semi-quantitative RT-PCR in response to recombinant mouse TNF-α. A dose-dependent upregulation of MT1-MMP expression in WT mice (approximately six-fold) was found, while MT1-MMP expression in WR mice remained unchanged (Fig. 6).

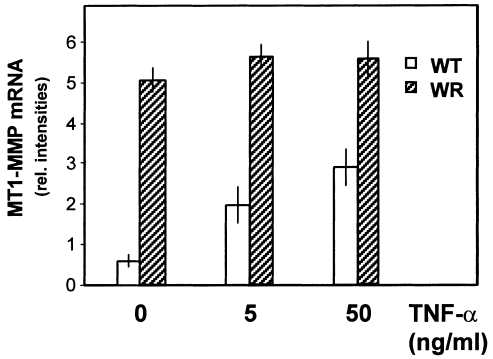

Fig. 6. Levels of MT1-MMP mRNA in primary astrocyte cultures from WT and WR mice in response to TNF-α. Astrocyte cultures were treated for 24 h with recombinant mouse TNF-α at the indicated concentrations. The mRNA levels of MT1-MMP were determined by radioactive hybridization of PCR products (see Section 2). Mean values of three independent experiments ± S.D.

4. Discussion

In the CNS, an increase in active MT1-MMP and MMP-2 with subsequent local ECM degradation is a key event for tumor progression and invasion of malignant gliomas. In addition, altered levels of MMPs and TIMPs have been implicated in CNS diseases, such as Alzheimer disease and multiple sclerosis [2]. Neuroinflammatory signals affect the level of

TIMP-1 and MT1-MMP in transgenic animal models expressing TNF-α under the control of the astrocyte specific GFAP promotor [27]. However, the role of MT1-MMP and TIMPs in progressive neurodegenerative diseases has been elusive.

We show that concomitantly with neurodegeneration in the CNS of WR mice, MT1-MMP expression is upregulated 3–5-fold at the mRNA level. MT1-MMP specific antibodies stained reactive astrocytes in the WR SC but not microglia. In contrast, in Alzheimer's disease brain white matter microglia express MT1-MMP [28]. These results might reflect divergent pathways for microglia activation in different neurological disorders.

Immunoblot analysis of CNS extracts revealed a major band of 60 kDa which most likely corresponds to the active form of MT1-MMP [7]. Comparison of the immunofluorescence staining using α-HD with α-PD revealed a higher labeling with the α-HD antibody, which recognizes both the pro- and active form. Therefore, a major proportion of MT1-MMP detected in cryosections corresponds to the processed form, although a significant amount represents the proform. The difference in the amount of proform detected in plasma membrane extracts and cryosections may in part be due to partial degradation during preparation of plasma membrane fractions despite the presence of protease inhibitors. A much higher proportion of active MT1-MMP has been detected in human astrocytic tumors and C6 glioma cells which were associated with enhanced MMP-2 processing activity [29,30]. In our study, we found both an increased cell-associated proteolytic activity of WR astrocytes as compared to WT, and an increase in the amount of secreted MMPs. Thus, the enhanced expression of MT1-MMP in WR astrocytes correlates with an increase in secreted activated MMPs.

Along with upregulated MT1-MMP expression, TIMP-1 mRNA levels were drastically increased in WR SC and BS, while TIMP-2 was constitutively expressed in both WT and WR mice. In WR mice, there was a regional difference in the expression pattern of TIMP-1 and TIMP-3 with a higher increase in TIMP-3 mRNA levels in the Cbl and BS compared to the SC. Our results on TIMP expression suggest that TIMP-1 and TIMP-3 may regulate the activities of different MMPs in distinct regions of the CNS. Unlike TIMP-1 and TIMP-2, TIMP-3 is stored in the ECM [31]. Because TIMP-3 like TIMP-2 [32] is capable to control the activity of MT1-MMP, it could also provide a control of the activities of secreted MMPs as well as of MT1-MMP. Furthermore, TIMP-3 has been shown to inhibit ADAM10 and the TNF-α processing enzyme TACE [33,34]. The proinflammatory cytokine TNF-α is highly expressed in reactive glia in WR CNS compared to WT mice [13] and the MT1-MMP expression of primary astrocyte cultures from WT is strongly induced by TNF-α, whereas MT1-MMP expression of WR-derived astrocytes cannot be further augmented in response to TNF-α, presumably because it is already activated. These results point to TNF-α as an important regulator of astrogliosis and associated MT1-MMP overexpression [13]. Furthermore, TNF-α is a known inductor for TIMP-1 expression in astrocytes and brain endothelial cells [26]. TIMP-1 has been reported to control the activity of MMP-3 and MMP-9 [35,36]. In normal adult mice, TIMP-1, MMP-3 and MMP-9 expression in the brain is low, but upregulated in response to tissue injury, inflammation and malignant tumors [27,37–39]. The novel finding is that in addition to TIMP-1, also TIMP-3 is transcriptionally upregulated in a neurodegenerative disease. Compared to TIMP-2 mRNA levels and to the expression profiles in CNS extracts, TIMP-1 and TIMP-3 expression in primary astrocytes from both WR and WT mice was relatively low. Thus, reactive astrocytes are probably not the main source of the elevated TIMP-1 and TIMP-3 expression, although we cannot rule out that primary astrocyte cultures cease to produce TIMPs.

In primary astrocyte cultures, MT1-MMP specifically localizes to outgrowing processes. MT1-MMP expression may thus facilitate the breakdown of glia inhibitory proteins, such as tenascin and the high molecular weight myelin protein NI-220, thus enabling astrocytes to have access to the sites of motoneuron degeneration. MT1-MMP by itself has been described to cleave various ECM components such as laminin-5 and collagen [40]. It has recently been shown that the migration of epithelia cells over laminin-5 is dependent on MT1-MMP expression [41]. MT1-MMP expression on reactive astrocytes may facilitate neuronal regeneration and aid axonal outgrowth.

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